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TITLE: Development of a Transgenic Mouse Model for Breast Cancer
that is Optimized for the Study of T Cell-Based
Therapeutic Strategies

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13. ABSTRACT (Maximum 200 Words) Our goal is to develop a transgenic mouse model for breast cancer that will allow the <i>in vivo</i> activities of tumor-specific T cell clones to be tracked at all stages of tumorigenesis and after various immune interventions. We proposed to "tag" the <i>neu</i> oncogene with two defined T cell epitopes so as to confer recognition by available T cell receptor (TCR) transgenic T cells. When expressed as a transgene in mammary epithelium, epitope-tagged <i>neu</i> (designated <i>neu</i> ^{OT1/OT2}) should induce formation of mammary adenocarcinomas that express the epitope tags and hence are recognizable by adoptively transferred TCR transgenic T cells. We have generated three <i>neu</i> ^{OT1/OT2} transgene-positive founder lines, and expression of <i>neu</i> ^{OT1/OT2} in mammary epithelium has been confirmed by Northern blot and by an immunological response to the epitope tag. Unfortunately, no mice have yet developed tumors, therefore the <i>neu</i> ^{OT1/OT2} mice are being crossed to mutant p53 and SV40 Middle T Antigen transgenic mice to accelerate mammary tumorigenesis. Meanwhile, we have used a transplantable lymphoma model to study the T cell proliferative response to tumors versus conventional antigenic stimuli. Furthermore, we have investigated the phenomenon of "epitope spreading" in the lymphoma model as a means to eradicate antigen-negative tumor cell variants.				
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BC990655 Annual Progress Report, June 2003

PI: Brad H. Nelson, Ph.D.

Title of Project: Development of a transgenic mouse model for breast cancer that is optimized for the study of T cell based therapeutic strategies

Introduction:

Currently, the development of immune-based therapies for breast cancer is impeded by the lack of an animal model that both mimics spontaneous human disease and is amenable to detailed monitoring of the activities of multiple, defined T cell clones that recognize tumor antigens. In this project, we are creating transgenic mice that are genetically programmed to develop spontaneous mammary tumors expressing defined T cell epitopes. Once such mice are available and tumors have developed, we will adoptively transfer CD4+ and CD8+ T cell clones with specificity for the engineered epitopes. These T cells will then be tracked in vivo and analyzed for functional responses to tumor cells. In future, this system will be used to evaluate the mechanisms and efficacy of immune-based therapeutic and preventative strategies. The specific aims of this proposal are:

- (1) To construct a dual epitope-tagged version of the oncogene *neu* that is recognized by two epitope-specific T cell clones while retaining transforming potential;
- (2) To assess the ability of epitope-tagged *neu*, upon expression in transgenic mice, to induce the formation of mammary tumors that are recognized by epitope-specific CD4+ and CD8+ T cell clones.

Body:

Aim 1: To construct a dual epitope-tagged version of the oncogene *neu* that is recognized by two epitope-specific T cell clones while retaining transforming potential.

Aim 1 was completed in its entirety during the first year of funding, as described in the 2001 Annual Report.

Aim 2: To assess the ability of epitope-tagged *neu*, upon expression in transgenic mice, to induce the formation of mammary tumors that are recognized by epitope-specific CD4+ and CD8+ T cell clones.

As described in previous annual reports, we have successfully generated three C57Bl/6 founder lines that stably transmit the *neu*^{OT1/OT2} transgene. This year, we demonstrated expression of the transgene by Northern blot. For each transgenic line, mammary tissue was harvested from two lactating females and subjected to standard Northern blot analysis using a probe corresponding to the *neu*^{OT1/OT2} cDNA. Tissue from a transgene-negative littermate served as a negative control. All three founder lines were found to express the *neu*^{OT1/OT2} mRNA, whereas control tissue was negative for expression, as expected. All tissue samples were positive for expression of the housekeeping gene GAPDH.

Expression of the *neu*^{OT1/OT2} transgene at the protein level was demonstrated indirectly by assessing the proliferative response of CD8+ OT-I T cells after adoptive transfer into two transgene-positive females. The premise underlying this experiment is that OT-I T cells should proliferate upon recognition of the OT-I epitope tag encoded within *neu*^{OT1/OT2}. T-cell proliferation was measured by pre-staining T cells with the fluorescent dye CFSE and measuring incremental loss of the dye after cell division had occurred in vivo. For a negative control, OT-I T cells were also infused into transgene-negative mice. We observed clear proliferation of OT-I T cells in transgene-positive, but not transgene-negative, mice. Therefore, the *neu*^{OT1/OT2} transgene is expressed at both the mRNA and protein level in these mice.

Unfortunately, none of the female founders or their offspring have developed mammary tumors. The oldest transgene-positive females that have been monitored are now almost two years of age. Even if tumors

were to arise in these mice in the near future, this is an unacceptably long latency period. Therefore, we have undertaken two alternative strategies to try to decrease the latency of tumor formation:

(1) Some founders are being bred to mice that express a dominant-negative p53 transgene in mammary epithelium (under the control of the whey acidic protein [WAP] promoter). This dominant-negative form of p53 has been shown by other groups to greatly accelerate *neu*-induced tumorigenesis. To date, we have generated 3 doubly transgenic pups (i.e., *neu*^{OT1/OT2} x WAP/p53), with several more litters on the way.

(2) Other founders are being bred to mice that express the SV40 middle T antigen under the control of the MMTV promoter. This transgene has been shown by other groups to strongly induce mammary tumors on the C57Bl/6 background. To date, we have generated several litters of potentially doubly transgenic pups (i.e., *neu*^{OT1/OT2} x MMTV/mTag), and these are currently being genotyped.

While we wait for our transgenic mice to develop tumors, we have made considerable progress with adoptive T cell transfer experiments using a transplantable lymphoma model, similar to the experiments that were described in Aim 2 of the original proposal. We have used the well-characterized lymphoma model involving the EL-4 cell line transfected to express the model antigen chicken ovalbumin (OVA). As reported last year, we find that OT-I T cells undergo a vigorous proliferative response to the EL-4/OVA tumor. Interestingly, these proliferating T cells fail to express the interleukin-2 (IL-2) receptor alpha chain (CD25), which implies that this is an IL-2-independent proliferative response. In the past year, we have extended these findings to show that the OT-I response to virally-delivered OVA (i.e., VSV-OVA) also occurs without expression of CD25. Moreover, we have shown that OT-I T cells that are genetically deficient in the IL-2 receptor beta chain (CD122) undergo a seemingly normal proliferative response to OVA in vivo and can also eradicate OVA-positive lymphomas (Figure 1). CD122 is an essential component of both the IL-2 and IL-15 receptors, therefore our results lead to the surprising conclusion that neither IL-2 nor IL-15 is required for the proliferative or anti-tumor activity of CD8+ T cells. These results challenge the wisdom of infusing IL-2 systemically to enhance cytolytic T cell responses, as is currently done for some melanoma and renal cancer patients.

The above results also raise the obvious question of which, if any, cytokines may drive CD8+ T cell proliferation in vivo. Since all known mitogenic cytokines operate through members of the STAT transcription factor family, we analyzed proliferating OT-I T cells by Western blot to see which of the 7 STAT molecules might be phosphorylated and hence activated. Consistent with the IL-2 receptor results described above, STAT5 (which is activated by IL-2 and IL-15) was not phosphorylated in these cells. Instead, the related transcription factors STAT1 and STAT3 were strongly phosphorylated (Figure 2). This suggests that a cytokine that activates STAT1 and STAT3 may drive T cell proliferation. This narrows the search to approximately 10 candidate cytokines, and these are being systematically tested by adding anti-cytokine inhibitory antibodies to the T cell cultures.

Finally, we have used the EL4/OVA system to study the phenomenon of antigen spreading. A major concern with antigen-specific immunotherapy is the potential to select for the outgrowth of variant tumor cells that have lost expression of the target antigen (so called "antigen-loss variants"). This concern would be ameliorated if the immune response were to spontaneously spread to other antigens expressed by the tumor. Indeed, antigen spreading has been documented in many autoimmune diseases, and is generally associated with progressive tissue destruction. In the past year, we have found that the adoptive transfer of OT-I and OT-II T cells not only eradicates OVA-positive tumors (as might be expected given that OT-I and OT-II T cells recognize ovalbumin), but also primes mice to later reject a challenge of EL4 tumor cells that lack expression of OVA. This suggests that the initial immune response to OVA later spreads to other unknown antigens expressed by the EL4 tumor. We are currently trying to better define the kinetics and immunological requirements for antigen spreading in this system, and we are also considering ways to potentially identify the novel EL4 antigens that are being recognized in these mice. Ultimately, we intend to also address these issues in the mammary tumor model.

Key Research Accomplishments:

The following items have been completed or are underway:

Task 1. To construct a dual epitope-tagged version of the oncogene *neu* that is recognized by two epitope-specific T cell clones while retaining transforming potential (Months 1-12). ***completed**

- Construct plasmids encoding single (*neu*^{IE α} and *neu*^{OVA}) and dual (*neu*^{IE α /OVA}) epitope-tagged versions of *neu*; verify DNA sequence (Months 1-3). ***completed**
- Evaluate signaling and transforming properties of epitope-tagged and untagged versions of *neu* in cell lines; if problems noted, modify epitopes as needed (Months 4-12). ***completed**
- In vitro assays to evaluate recognition of IE α and OVA epitopes by CD4+ and CD8+ T cells from TCR-transgenic mice (Months 4-12). ***completed**

Task 2. To assess the ability of epitope-tagged *neu*, upon expression in transgenic mice, to induce the formation of mammary tumors that are recognized by epitope-specific CD4+ and CD8+ T cell clones (Months 13-36). ***underway**

- Construct MMTV vector with human growth hormone gene at 3' end into which to introduce *neu* transgenes (Months 9-12). ***completed**
- Insert untagged (*neu*) and dual tagged (*neu*^{IE α /OVA}) cDNAs into MMTV vector (Month 13). ***completed**
- Provide transgenes to the Dept. of Immunology at the University of Washington and have C57Bl/6 transgenic founder mice generated (Months 14-17). ***completed**
- Perform PCR on tail DNA of pups (approximately 60 animals); breed transgene-positive founders (10-12 animals) (Months 18-19). ***completed**
- Expect birth of F2 generation (50-100 animals); perform PCR on tail DNA; cull males (Month 20). ***completed**
- Monitor female F2 mice for tumor formation (25-50 animals); as tumors develop, perform autopsies and tumor histology with the Dept. of Comparative Medicine at the Univ. of Washington (Months 22-32). ***underway – alternative strategies using mutant p53 and middle T antigen transgenes have been initiated**
- Perform adoptive T cell transfer experiments on animals that develop tumors (approximately 35 *neu* mice, 35 *neu*^{IE α /OVA} mice, 20 IE α -specific TCR transgenic mice, and 20 OVA-specific TCR transgenic mice (Months 30-36). ***underway using a lymphoma model**

Reportable Outcomes:

Posters:

Identifying the signaling pathways that drive T-cell proliferation in response to tumors. Ryan M. Teague, Richard M. Tempero, and Brad H. Nelson. Era of Hope Department of Defense Breast Cancer Research Program Meeting, Orlando, FL, September 2002.

Primary in vivo expansion of naïve CD8⁺ T cells in the absence of IL-2 receptor and STAT5 signaling. Ryan M. Teague, Richard M. Tempero, and Brad H. Nelson. Annual Meeting of the American Association of Immunologists, Denver CO, 2003.

Invited presentations:

Identifying the signaling pathways that drive T-cell proliferation in response to tumors. Brad H. Nelson. 10th Annual SPORE Investigators Workshop, Chantilly VA, July 2002.

The immune response to cancer. Brad H. Nelson. Annual Meeting of the British Columbia Cancer Agency, Vancouver, BC, Canada, November 2002.

The immune response to cancer. Brad H. Nelson. Fox Chase Cancer Center, Philadelphia PA, November 2002.

Career advancement:

The PI, Brad Nelson, has been appointed Director of the Research Laboratories for the British Columbia Cancer Agency's Vancouver Island Centre (Victoria, BC). His work in this animal tumor model was integral to his success in this competition. He will be moving there on July 1, 2003. (The DOD has already been informed of this move.)

Conclusions:

The mouse model we are developing should lead to an improved understanding of the immune response to breast cancer and may facilitate the development of novel immune-based therapies or immunopreventive strategies for this disease. Toward this goal, we have now created a dually epitope-tagged version of *neu* that is recognized by the appropriate CD4+ and CD8+ T cells while retaining transforming potential. Three transgenic founder lines have been established that express this version of *neu* in mammary epithelium. Expression of epitope-tagged *neu* has been demonstrated by Northern blot and by an immunological response of OT-I T cells to the epitope tag. Alternative strategies involving mutant p53 and SV40 middle T antigen transgenes have been initiated to decrease the latency of tumor formation in this model. Meanwhile, adoptive T cell studies are underway using a convenient transplantable lymphoma model. With this model, we have discovered that CD8+ T cell proliferation in vivo occurs independent of the IL-2 or IL-15 receptors, and may involve an unknown cytokine that activates the transcription factors STAT1 and STAT3. Finally, we have shown that the lymphoma model can be used to study the phenomenon of antigen spreading, which may be an essential component of successful immunotherapies for cancer. In Year 4, we will continue to pursue the goals outlined in Aim 2 of our original proposal, and hope to successfully decrease the latency of tumor formation in our model so that adoptive transfer experiments can be performed using spontaneous mammary tumors. No other changes to the research plan are expected.

References:

None.

Appendices:

See accompanying Figures 1 and 2.

Figure 1. CD8+ T cells that lack functional IL-2 and IL-15 receptors proliferate normally in vivo in response to cognate antigen. Normal C57Bl/6 mice were infused by tail vein injection with 5×10^6 naive OT-I TCR transgenic T cells that were either wildtype or genetically deficient for the IL-2R beta chain (CD122). T cells were pre-labeled with the fluorescent dye CFSE. On the same day, mice were injected with 1 mg soluble OVA (right side of each group) or left untreated (left side of each group). Three days later, lymphocytes were isolated from lymph nodes draining the tumor bed, stained with PE-conjugated anti-CD8, and analyzed by flow cytometry. CFSE intensity is shown on the "FL1" axis. Proliferating T cells can be identified by their diminishing CFSE intensity with each cell division (circled populations of cells in upper panels). Expression of the IL-2R alpha (CD25) and beta (CD122) chains was assessed using specific antibodies (FL2 axis, lower panels). The results indicate that OT-I cells proliferate vigorously in response to OVA, irrespective of IL-2R beta expression.

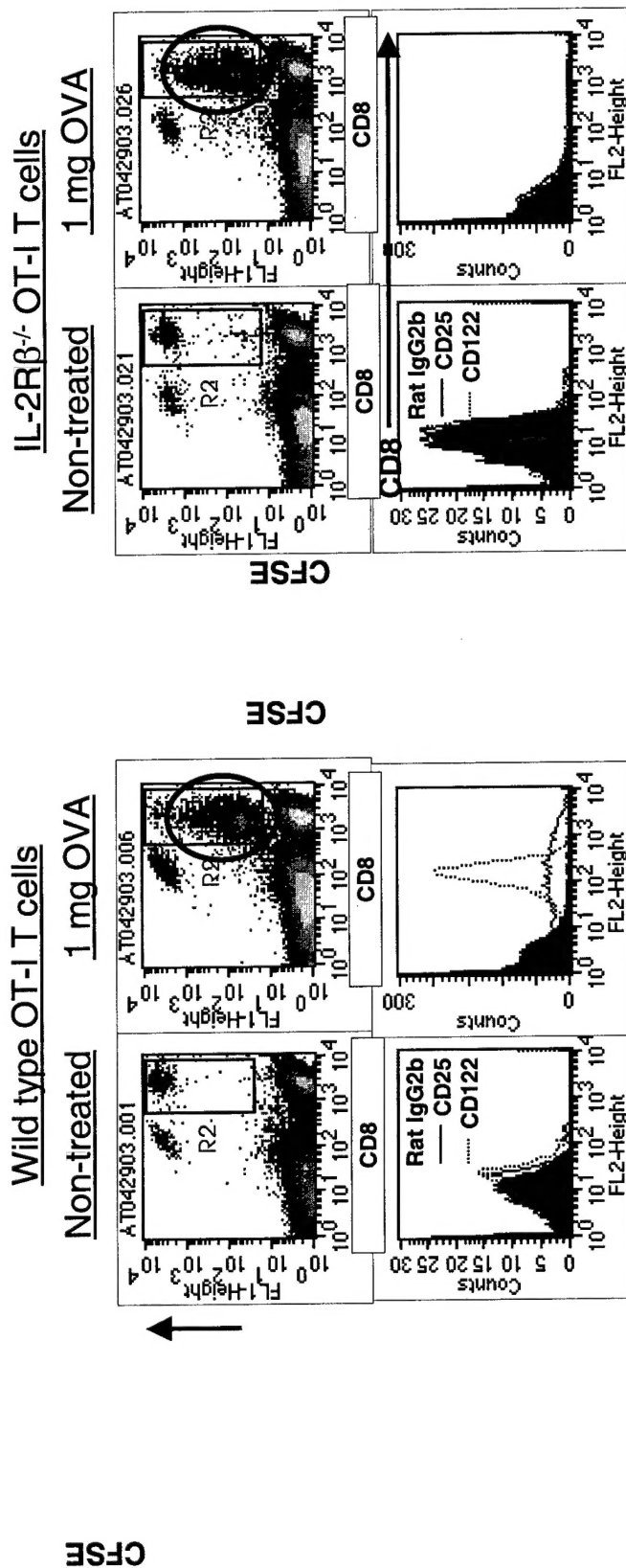


Figure 2. Proliferating CD8⁺ T Cells express phospho-STAT1 and STAT3 but no detectable phospho-STAT4, STAT5 or STAT6. Naive OT-I TCR transgenic T cells were stimulated in vitro with cognate antigen (OVA) for three days. T cells were then harvested and cytoplasmic fractions were analyzed by Western blot using the indicated phospho-specific antibodies. Where indicated, some T cells were given a 30 minute pulse of IL-2 just before harvesting to stimulate phosphorylation of STAT5 and thereby provide a positive control. The left panels show results for four different STAT molecules, as well as ERK, which is activated by antigen alone and serves as a positive control. The right panels show a time course of phosphorylation for STAT1, STAT3, STAT5 and ERK after stimulation with OVA.

